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## Branched-Chain Amino Acid Transport in Cytoplasmic Membranes of *Leuconostoc mesenteroides* subsp. *dextranicum* CNRZ 1273

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Membrane vesicles of *Leuconostoc mesenteroides* subsp. *dextranicum* fused with proteoliposomes prepared from *Escherichia coli* phospholipids containing beef heart cytochrome *c* oxidase were used to study the transport of branched-chain amino acids in a strain isolated from a raw milk cheese. At a medium pH of 6.0, oxidation of an electron donor system comprising ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and horse heart cytochrome *c* resulted in a membrane potential ( $\Delta\psi$ ) of  $-60$  mV, a pH gradient of  $-36$  mV, and an L-leucine accumulation of 76-fold ( $\Delta\mu_{\text{Leu}}/F = 108$  mV). Leucine uptake in hybrid membranes in which a  $\Delta\psi$ ,  $\Delta\text{pH}$ , sodium ion gradient, or a combination of these was imposed artificially revealed that both components of the proton motive force ( $\Delta p$ ) could drive leucine uptake but that a chemical sodium gradient could not. Kinetic analysis of leucine (valine) transport indicated three secondary transport systems with  $K_i$  values of 1.7 (0.8) mM, 4.3 (5.9)  $\mu\text{M}$ , and 65 (29) nM, respectively. L-Leucine transport via the high-affinity leucine transport system ( $K_i = 4.3$   $\mu\text{M}$ ) was competitively inhibited by L-valine and L-isoleucine ( $K_i$  and  $K_i$  values were similar), demonstrating that the transport system translocates branched-chain amino acids. Similar studies with these hybrid membranes indicated the presence of high-affinity secondary transport systems for 10 other amino acids.

*Leuconostoc* species are fastidious organisms that require a number of amino acids and vitamins for optimum growth. In rich media, most *Leuconostoc* strains grow more slowly than do other lactic acid bacteria. Growth in milk is usually poor and is enhanced by the addition of glucose and yeast extract (3). *Leuconostoc* strains are used as starter strains in dairy and other commercially developed fermentations, and the industrial application of these organisms can be developed further if the factors that limit growth are defined (3, 16, 17). The mechanisms and energetics of solute transport in *Leuconostoc* species have not yet been studied.

Studies of amino acid transport systems in other lactic acid bacteria like lactococci reveal the following: (i) growth of these organisms depends on an exogenous supply of amino acids and/or peptides; (ii) primary and secondary amino acid transport systems are present that are driven by phosphate-bond energy, a proton motive force, or sodium motive force or mediated in antiport with other amino acids; and (iii) the rate of growth can be directly affected by the rate of uptake of certain amino acids or peptides (7, 14, 19, 21).

The characterization of lactococcal amino acid transport systems has been facilitated by the use of membrane vesicles. By fusing membrane vesicles with liposomes prepared from bacterial phospholipids, hybrid membranes can be obtained for which the ion and acid permeability is lower than that for membrane vesicles (7). Furthermore, the incorporation of proton pumps such as cytochrome *c* oxidase or bacterial reaction centers into the liposomes makes it possible to incorporate proton motive force-generating systems in these hybrid membranes (9–11).

In this study, *Leuconostoc* cytoplasmic membranes were used to characterize branched-chain amino acid transport kinetics, specificity, and energetics. The energetics of the

uptake of these and other amino acids are discussed in relation to those properties found in *Lactococcus* strains.

### MATERIALS AND METHODS

**Cell growth and membrane vesicle preparation.** *Leuconostoc mesenteroides* subsp. *dextranicum* CNRZ 1273, a raw milk French cheese isolate, was obtained from the CNRZ culture collection, Jouy-en-Josas, France. For membrane vesicle preparation, 5-liter batch cultures were grown anaerobically at 30°C in commercial MRS broth (Difco) at a constant pH of 6.5 until the late-exponential phase. The cells were centrifuged, washed, and concentrated, and membrane vesicles were prepared as previously described (18), except that 2,000 U of Mutanolysin (Sigma) was added to enhance cell lysis. The isolated membrane vesicles were frozen in liquid nitrogen and stored at  $-20^\circ\text{C}$  in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 to 25 mg of protein per ml and thawed just before use.

**Purification of bacterial phospholipids and liposome preparation.** Acetone-ether-washed crude *Escherichia coli* phospholipid (L-phosphatidylethanolamine, type IX; Sigma) stored in chloroform was concentrated and desolvated in *N*-octylglucoside. Liposomes were prepared by dialysis as previously described (13). Where indicated, beef heart mitochondrial cytochrome *c* oxidase was incorporated into the liposomes by adding the purified enzyme (23) at a concentration of 11 nmol of heme per 100 mg of phospholipid just before dialysis.

**Fusion of liposomes with membrane vesicles.** After dialysis, the liposomes were mixed with a membrane vesicle suspension at a concentration of 10 mg of phospholipid per mg of vesicular protein and immediately frozen in liquid nitrogen. The two components were fused by being thawed slowly at room temperature and then sonication for 8 s in a plastic tube with a probe-type sonicator at full amplitude (8, 10, 11). The resulting hybrid membranes were concentrated or washed

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by centrifugation in a Beckman air-driven ultracentrifuge for 60 min at a pressure of 1.6 bar (ca.  $1.6 \times 10^5$  Pa).

**Amino acid uptake studies.** Amino acid uptake driven by a proton motive force ( $\Delta p$ ) generated by cytochrome *c* oxidase activity was studied in an assay mixture composed of 30 to 60  $\mu$ g of protein from the hybrid membranes in 200  $\mu$ l at 30°C. Assay components included well-aerated 100 mM potassium phosphate or 100 mM morpholineethanesulfonic acid (MES)–KOH (pH 6.0) and an electron donor system comprising cytochrome *c* (20  $\mu$ M), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (1 mM), and ascorbate (10 mM, pH 6.0). After 2 min of continuous aeration, labeled amino acid was added to start the uptake reaction. The reaction was stopped by adding 2 ml of ice-cold 0.1 M LiCl and filtering immediately over 0.45- $\mu$ m-pore-size cellulose nitrate filters (type HAWP; Millipore Corp.). The filters were then washed with 2 ml of 0.1 mM LiCl, dried, and placed in 5 ml of scintillation fluid, and radioactivity was counted with a Beckman model LS 2800 liquid scintillation counter.

The amino acid uptake driven by artificial gradients was studied in hybrid membranes devoid of cytochrome *c* oxidase. These hybrid membranes were suspended in 100 mM MES–KOH or 20 mM MES–KOH–80 mM potassium acetate (pH 6.0) and loaded with potassium by incubation with 2 nmol of valinomycin per mg of membrane protein for 1 h on ice. The membranes were concentrated by centrifugation. Samples containing 40 to 60  $\mu$ g of membrane protein were diluted 50-fold with 200  $\mu$ l of specified buffers containing radioactively labeled amino acid. Hybrid membranes suspended in MES–potassium acetate were diluted into 100 mM MES–choline (pH 6.0) to generate a membrane potential and pH gradient or into 100 mM MES–KOH (pH 6.0) to establish a pH gradient. Hybrid membranes suspended in MES–KOH were diluted into 100 mM MES–NaOH (pH 6.0) to establish a membrane potential and a chemical gradient of sodium ions. A chemical gradient of sodium ions alone was generated by diluting hybrid membranes in 100 mM MES–KOH (pH 6.0) (no valinomycin treatment) 50-fold into 100 mM MES–NaOH (pH 6.0). Transport assays were carried out at 24°C as described above. The amino acids were added at the following concentrations: methionine, 1.7  $\mu$ M; valine, isoleucine, lysine, and phenylalanine, 1.8  $\mu$ M; glutamate, 2.2  $\mu$ M; threonine, 2.5  $\mu$ M; tyrosine, 2.6  $\mu$ M; alanine, 3.1  $\mu$ M; tryptophan, 3.4  $\mu$ M; serine, 3.5  $\mu$ M; glycine, 5.3  $\mu$ M; and arginine, 5.8  $\mu$ M.

For kinetic experiments, the initial rates of uptake of leucine (at leucine concentrations ranging from 7 nM to 2 mM) were determined after 15 s in the presence of an artificially imposed  $\Delta p$ . The  $\Delta p$  was initiated by diluting potassium-loaded membranes suspended in MES–potassium acetate (pH 6.0) into MES–choline (pH 6.0).

The effects of inhibitors on the high-affinity leucine uptake system were studied under the same conditions as for the kinetic experiments at a fixed leucine concentration of 3  $\mu$ M. The apparent inhibitor constants ( $K_i'$ ) were calculated as described previously (20).

**Measurement of the membrane potential and pH gradient.** The membrane potential was determined in hybrid membranes under the conditions described above for cytochrome *c* oxidase-driven uptake with a tetraphenylphosphonium ion-selective electrode. Hybrid membranes (0.3 mg of protein) were added to MES–KOH (pH 6.0) containing 4  $\mu$ M tetraphenylphosphonium ion to a final volume of 1 ml. A zero value of the membrane potential ( $\Delta\psi$ ) was obtained by adding valinomycin at a concentration of 400 nM to the incubation mixture. The membrane potential was calculated

by applying the Nernst equation and corrected for probe binding (15).

The pH gradient was measured from the change of the fluorescence intensity of entrapped pyranine (100  $\mu$ M) (5, 10). Dissipation of the pH gradient was achieved by adding nigericin at a concentration of 100 nM.

**Materials.** The uniformly  $^{14}$ C-labeled amino acids used in this study were obtained from either Amersham Corp., Buckinghamshire, England, or Commissariat à l'Energie Atomique, Saclay, France: L-leucine, 11.4 TBq/mol; L-valine, 10.4 TBq/mol; L-isoleucine, 10.9 TBq/mol; L-alanine, 5.9 TBq/mol; glycine, 1.8 TBq/mol; L-serine, 5.6 TBq/mol; L-threonine, 7.4 TBq/mol; L-methionine, 10.1 TBq/mol; L-glutamic acid, 9.3 TBq/mol; L-lysine and L-arginine, 11.1 TBq/mol; L-phenylalanine, 16.6 TBq/mol; L-tyrosine, 14.4 TBq/mol. Other amino acids from the same sources include L-[ring-2- $^{14}$ C]tryptophan (1.8 TBq/mol) and L-[4,5- $^3$ H]leucine (5.0 TBq/mol).

## RESULTS

**Energetics of high-affinity branched-chain amino acid uptake.** Hybrid membranes of *L. mesenteroides* subsp. *dextranum* CNRZ 1273 fused with liposomes containing cytochrome *c* oxidase generated at pH 6.0, in the presence of ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, and cytochrome *c*, a membrane potential of  $-60$  mV and a pH gradient of  $-36$  mV. Under these conditions a high rate of leucine uptake was observed, and a steady-state accumulation level of 76-fold (108 mV) was achieved (Fig. 1). The ionophores valinomycin and nigericin were used to investigate the nature of the driving force for branched-chain amino acid uptake. Under conditions of amino acid uptake, valinomycin (0.8  $\mu$ M) dissipated the membrane potential as indicated from the distribution of tetraphenylphosphonium ion, whereas nigericin (0.8  $\mu$ M) dissipated the pH gradient as shown by the change in fluorescence of entrapped pyranine. Valinomycin and nigericin individually inhibited the rate of leucine uptake by about 40% (Fig. 1). Complete inhibition of leucine transport was observed when the total  $\Delta p$  was dissipated by the addition of both ionophores. Similar results were obtained when valine transport was assayed in the hybrid membranes (data not shown).

To verify that both components of the  $\Delta p$  function as the driving force for leucine uptake, hybrid membranes devoid of cytochrome *c* oxidase were subjected to artificial gradients of protons, potassium ions, sodium ions, or combinations thereof. Figure 2 shows the effects of the established gradients on leucine uptake. In the presence of both a membrane potential and a pH gradient, the highest initial rate of leucine uptake was obtained. In the presence of either a membrane potential or a pH gradient, the initial rate was reduced about 40%. Sodium ions had little or no effect on  $\Delta\psi$ - or  $\Delta p$ H-driven leucine uptake. Also, the presence of a transmembrane gradient of sodium ions on top of a  $\Delta\psi$  did not effect the uptake of leucine. In the absence of any gradient or in the presence of a sodium gradient alone, negligible rates of leucine uptake were observed.

**Kinetic analysis of  $\Delta p$ -driven branched-chain amino acid uptake.** The kinetics of artificially imposed  $\Delta p$ -driven leucine uptake in hybrid membranes without cytochrome *c* oxidase was determined to obtain information about the different systems involved in the uptake. Multiple systems were observed from Woolf plots (Fig. 3), including a low-affinity component with an apparent  $K_t$  of 1.7 mM, a high-affinity component with a  $K_t$  of 4.3  $\mu$ M, and a very high-affinity

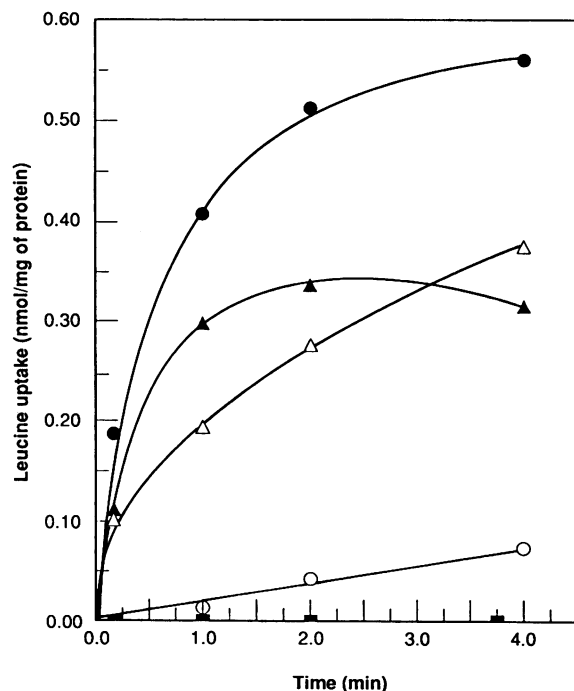


FIG. 1. Effect of the ionophores valinomycin and nigericin on leucine uptake by cytochrome *c* oxidase-containing hybrid membranes of *L. mesenteroides* subsp. *dextranicum* CNRZ 1273. Valinomycin and nigericin were added to a final concentration of  $0.8 \mu\text{M}$  2 min before radioactively labeled leucine was added. Symbols: ●, presence of the electron donor system; ○, absence of the electron donor system; △, electron donor system plus valinomycin; ▲, electron donor system plus nigericin; ■, electron donor system plus valinomycin and nigericin.

component with a  $K_i$  of 65 nM. The kinetics of  $\Delta p$ -driven valine uptake displayed the same profile from which  $K_i$  values of 0.8 mM,  $5.9 \mu\text{M}$ , and 29 nM, respectively, could be estimated (data not shown).

**Specificities of the transport systems.** The  $\Delta p$ -driven high-affinity leucine uptake system was subject to inhibition by several amino acids, including leucine, valine, and isoleucine, and to a lesser extent by cysteine, methionine, threonine, and phenylalanine (Table 1). High-affinity valine uptake was also strongly inhibited by all three branched-chain amino acids.

The three branched-chain amino acids competed more effectively with leucine than did all of the other amino acids tested; the inhibition was competitive (data not shown). The  $K_i'$  values for leucine, valine, and isoleucine were 6.3, 16.8, and  $10.5 \mu\text{M}$ , respectively, with L-[ $^{14}\text{C}$ ]leucine as a substrate (Table 1). These  $K_i'$  values approximated the  $K_i$  values obtained for leucine ( $4.3 \mu\text{M}$ ), valine ( $5.9 \mu\text{M}$ ), and isoleucine ( $10.5 \mu\text{M}$ ) high-affinity transport and the  $K_i'$  values determined from uptake experiments with L-[ $^{14}\text{C}$ ]valine as a substrate. Methionine and cysteine inhibited leucine transport to a lesser degree, with  $K_i'$  values of 128 and  $156 \mu\text{M}$ , respectively, indicating that the system has some affinity for non-branched-chain amino acids.

The low-affinity leucine uptake system was inhibited by valine and isoleucine with  $K_i'$  values of 3.4 and 3.5 mM, respectively (data not shown).

**Steady-state leucine accumulation ratio.** To obtain an estimate of the leucine concentration gradient and the leu-

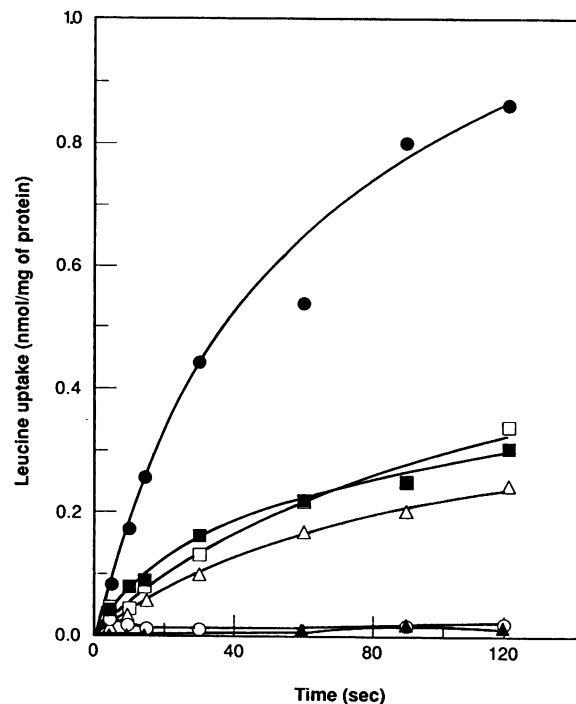


FIG. 2. Effect of artificially imposed ion gradients on leucine uptake in hybrid membranes of *L. mesenteroides* subsp. *dextranicum* fused with liposomes. The conditions for this experiment were as described in Materials and Methods. Symbols: ●, membrane potential and pH gradient; ○, no gradient; ■, pH gradient; □, membrane potential; ▲, sodium gradient; △, membrane potential and sodium ion gradient.

cine/ $\text{H}^+$  stoichiometric ratio during  $\Delta p$ -driven transport, steady-state accumulation ratios of leucine were determined in hybrid membranes containing cytochrome *c* oxidase. In the presence of the electron donor system ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine-cytochrome *c*, a constant  $\Delta p$  could be generated for more than 12 min. Steady-state leucine accumulation levels were reached after 4 min and maintained for several minutes thereafter. At leucine concentrations of 0.9 to  $9 \mu\text{M}$ , leucine accumulated at 76 to 38 times the external concentration. Under these conditions, mainly the high-affinity uptake system ( $K_i = 4.3 \mu\text{M}$ ) contributes to the accumulation of leucine. Leucine accumulation levels decreased at higher leucine concentrations, in accordance with an increase in the diffusion rate that can be expected at these higher concentrations (Fig. 4) (11). The first-order rate constant for leucine exit in the steady state ( $k_{ex}$ ), estimated from the initial rate of uptake and the internal amino acid concentration under steady-state conditions as described by Booth and Hamilton (2), was between 0.7 and  $0.9 \text{ min}^{-1}$ . The  $k_{ex}$  for leucine in the hybrid membranes compares to the values found in earlier studies, in which the first-order rate constant for passive diffusion of leucine contributed more than 60% of the  $k_{ex}$  (11). Due to passive and/or carrier-mediated efflux of leucine, the leucine concentration gradient ( $\Delta\mu_{\text{Leu}}/F$ ) could not achieve thermodynamic equilibrium with the  $\Delta p$ . By extrapolating the leucine accumulation ratio to an external leucine concentration of zero, the  $\Delta\mu_{\text{Leu}}/F$  could be estimated. From an analysis of the magnitude of  $\Delta p$  ( $-96 \text{ mV}$ ) and  $\Delta\mu_{\text{Leu}}/F$  ( $114 \text{ mV}$ ), the leucine/ $\text{H}^+$  stoichiometric ratio was estimated to

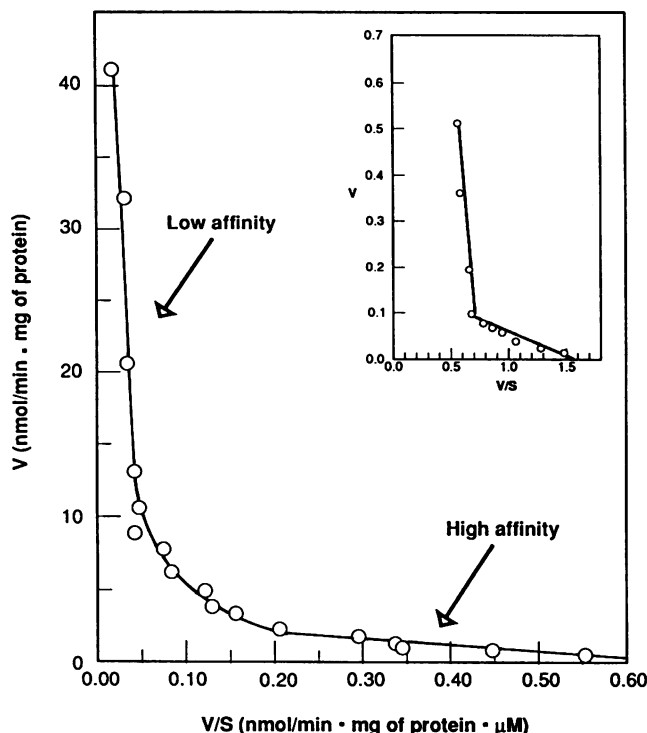


FIG. 3. Kinetics of artificially imposed  $\Delta p$ -driven leucine uptake in hybrid membranes of *L. mesenteroides* subsp. *dextranicum* 1273. Data are presented as Woolf plots. The inset shows the kinetics of very high-affinity leucine uptake in which high-specific-activity tritiated leucine (5.0 TBq/mmol) was used as a substrate.

be 1; i.e., at thermodynamic equilibrium,  $(n \times \Delta p) + (\Delta \mu_{\text{Leu}}/F) = 0$ .

**High-affinity amino acid transport.** The energetics of high-affinity amino acid uptake was investigated in hybrid membranes of *L. mesenteroides*. Uptake rates were measured in the presence of either a membrane potential and a pH gradient ( $\Delta p$ ) or a sodium gradient. Of the 14 amino acids tested, all amino acids except tryptophan were transported in the presence of a  $\Delta p$  (Table 2). Glutamate displayed the highest activity ( $9.1 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ ). However,

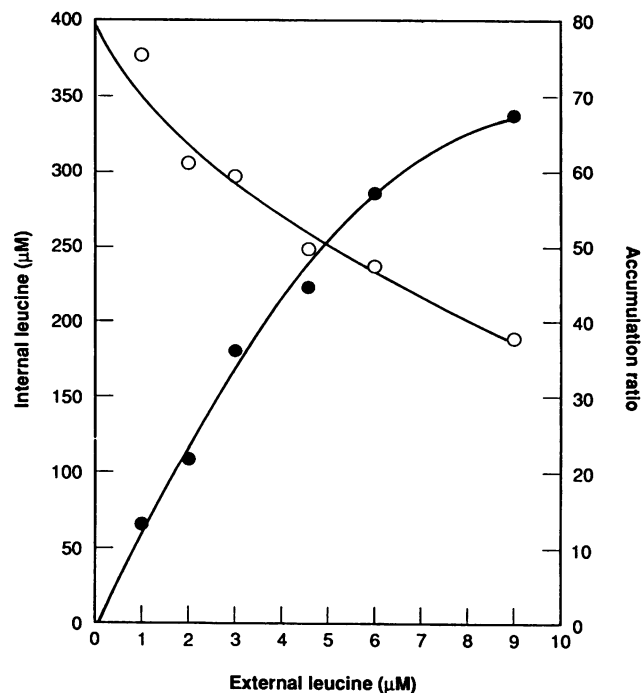


FIG. 4. Steady-state leucine accumulation in *L. mesenteroides* subsp. *dextranicum* hybrid membranes containing cytochrome *c* oxidase. Reaction mixtures were incubated for 5 min under a continuous stream of air and processed as described in the text for cytochrome *c* oxidase-driven uptake, i.e., in the presence of the electron donor system. Symbols: ●, internal leucine concentration; ○, accumulation ratio.

no transport activity was observed for any amino acid in the presence of a sodium gradient.

## DISCUSSION

Amino acid transport systems that are driven by a proton motive or sodium motive force have been analyzed in membrane vesicles of several bacteria (6, 9, 10–12, 22). This study also demonstrates that the amino acid transport systems of *L. mesenteroides* can be investigated in detail in

TABLE 1. Inhibition of high-affinity  $\Delta p$ -driven branched-chain amino acid transport in hybrid membranes of *L. mesenteroides* subsp. *dextranicum* CNRZ 1273

Inhibitor	$K_i'$ ( $\mu\text{M}$ )	
	L-Leucine	L-Valine
L-Leucine	6.3	13.6
L-Isoleucine	10.5	15.8
L-Valine	16.8	9.5
L-Methionine	128	281
L-Cysteine <sup>a</sup>	156	134
L-Threonine	307	359
L-Phenylalanine	505	ND <sup>b</sup>
L-Alanine, L-serine, glycine, L-arginine, L-lysine, L-histidine, L-proline	>1,000	ND

<sup>a</sup> No significant inhibition was observed when L-cysteine was replaced with the same concentration of dithiothreitol.

<sup>b</sup> ND, not determined.

TABLE 2. Artificially imposed  $\Delta p$ -driven high-affinity amino acid uptake in *L. mesenteroides* subsp. *dextranicum* CNRZ 1273

Amino acid	Uptake <sup>a</sup>
L-Glutamate	9.1
Glycine	4.4
L-Alanine	3.3
L-Threonine	3.1
L-Arginine	2.2
L-Serine	2.1
L-Leucine	1.9
L-Isoleucine	1.9
L-Phenylalanine	1.7
L-Valine	1.6
L-Lysine	1.5
L-Tyrosine	1.3
L-Methionine	0.6
L-Tryptophan	0.0

<sup>a</sup> Expressed as nanomoles of amino acid per minute per milligram of protein.

membrane vesicles and that a  $\Delta p$  of significant magnitude can be generated in membrane vesicles in which cytochrome *c* oxidase is incorporated by membrane fusion. These hybrid membranes proved to be excellent systems for detailed analysis of amino acid transport.

Kinetic analysis of leucine transport suggests that three distinct  $\Delta p$ -driven systems exist within the concentration range of 7 nM to 2 mM. The slope between the high- and low-affinity systems most likely results from the contribution of both high- and low-affinity components.

Growth of *L. mesenteroides* in milk with branched-chain amino acid concentrations in the low micromolar range (1, 5, 17) emphasizes the important role that the high-affinity leucine transport system would play during growth in this medium. In fermented milk or complex broths such as MRS (4), significantly higher concentrations of amino acids are present; consequently, the contribution of the low-affinity system in the uptake of leucine will be higher. Like the high-affinity system, the low-affinity  $\Delta p$ -driven leucine transport system appears to be specific for branched-chain amino acids.

Since a membrane potential and a pH gradient can drive uptake of leucine, whereas a gradient of sodium ions cannot, and since a collapse of the  $\Delta p$  completely abolishes leucine uptake, it is very likely that leucine is transported in symport with protons. Although the different leucine transport systems could not be studied separately, the analysis of the steady-state accumulation levels at micromolar concentrations of leucine (Fig. 4) and the magnitude of the  $\Delta p$  suggest that high-affinity leucine uptake occurs with a  $H^+$ /leucine stoichiometric ratio of 1.

There are several similarities between the high-affinity leucine transport systems of *L. mesenteroides* and *Lactococcus lactis* subsp. *cremoris* (8), namely, (i) the calculated  $K_t$  values (4.3 and 6.5  $\mu$ M, respectively); (ii) the high specificity for leucine, valine, and isoleucine; and (iii) the ability of the  $\Delta p$  to drive transport. However, only one branched-chain amino acid transport system has been detected in the *Lactococcus* species.

Preliminary results obtained from experiments in which the uptake of 11 other amino acids was studied indicate that the  $\Delta p$  and not a sodium gradient is used to drive the uptake of these amino acids in *L. mesenteroides*. Further characterization of the transport of these amino acids is required to determine the exact nature of these secondary transport systems.

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